Dot Blot Assay for Detection of Antidiacyltrehalose Antibodies in Tuberculous Patients

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A simple dot blot test with diacyltrehalose (DAT) as the antigen was developed to detect anti-DAT antibodies in tuberculous patients. To enhance antigen-antibody reaction detection, rabbit serum raised against human immunoglobulins was used prior to incubation with a protein A-colloidal gold complex. With the dot blot system, it was possible to obtain a sensitivity similar to that of enzyme-linked immunosorbent assay (ELISA) and a specificity of 97.14%, versus a specificity of 94.29% by the ELISA. We conclude that this simple and fast assay could be used in places where ELISA equipment is not easy available and that it might also be applicable with other *Mycobacterium tuberculosis* immunogenic antigens.

Tuberculosis is still a serious public health problem in the world, with about 8 million new cases per year and 3 million deaths; it has been estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (12). Approximately 95% of the cases are found in developing countries. Diagnosis of tuberculosis still relies on the detection of acid-fast bacilli by smear microscopy (Ziehl-Neelsen) and culturing of *M. tuberculosis* in the appropriate media. Although smear microscopy is rapid, culture is still the "gold standard" for diagnosis, but it takes about 6 to 8 weeks to complete. Because of that, efforts have been made to find better diagnostic procedures.

Methods that have been proposed to expedite the diagnosis of tuberculosis include the determination of mycobacterial DNA by PCR (13), the detection of *M. tuberculosis* antigens in biological samples (5, 21), and the measurement of the immune response. Of those methods, the most affordable for the clinical setting are the serological techniques, since they are easy to perform and require simple reagents. Many serological assays based on the enzyme-linked immunosorbent assay (ELISA) technique have been developed with immunogenic antigens from mycobacterium such as polysaccharides (22), proteins (6, 20), and glycolipids (7, 14, 23).

An ELISA with natural and synthetic glycolipids has been described to be useful in the serodiagnosis of tuberculosis and leprosy (14, 15). This test needed simplification to be useful in the field, where most diagnoses of tuberculosis are made. We decided, therefore, to develop a simple and rapid dot blot test for 2,3-di-*O*-acyltrehalose (DAT), a natural glycolipid, using a simple system in which a protein A-gold conjugate is enhanced with rabbit anti-human serum. The sensitivity and specificity of this method were compared to those of a conventional ELISA and the more sensitive ampELISA (26).

MATERIALS AND METHODS

Antigens. Fernand Papa (Unité de la Tuberculose et des Mycobactéries de L'Institut Pasteur, Paris, France) kindly provided the DAT antigen (formerly named sulfolipid IV). It was stored at −20°C as a stock solution of 1 mg/ml. The antigen was conserved in a chloroform-methanol (1:2) solution until use.

Sera. The serum samples were obtained with prior consent from patients attending the Hospital Universitario "Dr. José Eleuterio González" in Monterrey, Nuevo León, México. The sera were stored at −20°C and were sent to Ottawa, Ontario, Canada, where they were stored in aliquots at −70°C until use. A total of 44 samples were used in this assay. The samples were taken before the beginning of chemotherapy to avoid any modification of the immune response due to the elimination of microorganisms by the drugs. The patients were given a diagnosis of tuberculosis on the basis of clinical and radiological studies as well as by smear staining and culturing of sputum samples. As negative controls, 35 serum samples from healthy subjects with no previous symptoms of pulmonary disease were used. Thirty-one of them were obtained from students of the School of Medicine of the Universidad Autónoma de Neuvo León, and four were obtained from blood donors. The sera were stored under the same conditions described above and were tested less than 1 year from the time that they were taken.

Screening of solid supports. In order to select the best matrix support for the glycolipid, several commercial media were screened for spotting. We analyzed polysulfone (Tuffryn membrane filters HT-200 [pore size, 0.2 μm]; Gelman Sciences, Ann Arbor, Mich.), nylon (Zeta probe; Bio-Rad Laboratories, Richmond, Calif.), polyvinylidene difluoride (PVDF-plus [pore size, 0.45 μm]; MSI, Westboro, Mass.), and nitrocellulose (pore size, 0.45 μm; Bio-Rad) membranes. The assay was done by spotting several quantities of antigen in the different supports and incubating the supports with positive and negative sera by the methodology described below.

Dot blot assay. Since the chloroform-methanol-glycolipid solutions are corrosive for nitrocellulose paper, we evaporated 60 μl of this solvent and reconstituted it with 150 μl of hexane, which is harmless for the nitrocellulose paper. From this working solution, 2.5 μl was spotted onto nitrocellulose strips (approximately 10 by 4 mm), which were allowed to dry at room temperature. The strips were rinsed briefly in phosphate-buffered saline (PBS; pH 7.2) and were incubated overnight at 4°C in 5% skim milk (BBL) in PBS with 0.05% sodium azide to block the residual binding sites on the paper.

The strips were rinsed for 10 min in PBS and were then incubated with sera diluted 1:80 in 5% skim milk for 30 min at 37°C. After incubation, the strips were washed by the use of four changes of PBS and were further incubated for 10 min at 37°C with a rabbit anti-human serum (Dako-Immunoglobulins, Carpinteria, Calif.) diluted 1:500 in 5% skim milk. The rabbit serum reacts primarily with gamma, kappa, and lambda immunoglobulin G (IgG) chains. The strips were washed with PBS and were incubated with a protein A-gold complex (protein A labeled with 20-nm colloidal gold; A_{520} = 5.3; Sigma Chemical Co., St. Louis, Mo.) for 5 min at room temperature. The strips were washed four times and were allowed to dry on a filter paper. A clearly defined red spot at the site where the antigen was spotted was considered a positive result. A trace reaction or the absence of any reaction was considered a negative result.

ELISA. The ELISA method has been described before (14). Briefly, 25 μl of hexane containing 100 ng of glycolipid antigen was coated onto Dynatech Immulon-3 polystyrene microtiter plate wells, and the plates were allowed to dry at 37°C. Wells treated with hexane but not antigen were used to check for nonspecific serum absorption. After blocking by overnight incubation at 4°C with 5% bovine serum albumin (BSA) in PBS, the plates were washed with PBS. Test sera

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diluted 1:250 in 0.5% BSA in PBS were added in 100-μl volumes to each well. After 90 min of incubation followed by washing, goat anti-human IgG labeled with β-galactosidase (Biosys, Compiègne, France) was added to the wells, and the plates were incubated for 120 min. After additional washing, *o*-nitrophenyl-β-D-galactoside (Sigma) was added and the plates were incubated at 37°C for 60 min. The plates were read at 414 nm in a Titertek Multiskan MCC/340 reader (ICN Biomedicals); changes in A_{414} values were determined by subtracting the blank absorbance values from the test absorbance values and by using a correction factor obtained by making the absorbance in the wells containing the conjugate plus the substrate equal to a 100% response.

ampELISA. The amplified ELISA (ampELISA) was performed as reported previously (26). The plates were coated with 25 μl (50 ng) of a DAT working solution of 2 μg/ml in hexane and were allowed to dry at 37°C. After drying, 0.2 ml of 5% BSA in Tris-buffered saline (TBS) was added to each well, and the plates were incubated overnight at 4°C. The plates were washed twice with TBS, and 100 μl of the serum diluted 1:2,000 in 0.5% BSA in TBS was added to duplicate wells. After 1.5 h of incubation at 37°C, the plates were washed three times with TBS, 100 μl of a biotinylated anti-human IgG (gamma chain specific; Vector Laboratories, Burlingame, Calif.) diluted 1:5,000 in 0.5% BSA was added, and the plates were incubated for 2 h at 37°C. After washing four times with TBS, 100 μl of streptavidin-alkaline phosphatase conjugate (Bio-Rad) diluted 1:3,000 in 0.5% BSA was added to the wells, and the plates were incubated at 37°C for 30 min. The plates were washed five times with TBS, and 100 μl of substrate (0.25 mg of an NADP solution per ml in diethanolamine buffer–50 mM HCl [pH 9.5] with 1 mM $MgCl_2$ and 15 mM NaN_3) was added to each well. The plates were incubated at room temperature for 20 min, and 50 μl of the amplification solution containing 300 U of alcohol dehydrogenase (Boehringer Mannheim) per ml and 1.2 U of diaphorase (Boehringer Mannheim) per ml in 0.15% iodonitrotetrazolium in 0.025 M Na_2PO_4 buffer (pH 7.0 to 7.2) (to which 3% ethanol was added) was added to each well. The amplification reaction proceeded for 5 min. The reaction was stopped by the addition of 50 μl of 0.3 M H_2SO_4, and the optical density (OD) at 492 nm was determined.

RESULTS

Selection of the matrix support. In order to determine the best support for the testing of the glycolipid antigen, we assayed several matrices. We observed that under the conditions of this study only nitrocellulose paper presented a spot when it was used to test a positive serum sample. The other matrices showed no signal, reflecting perhaps the lack of binding of the glycolipids to the membranes. Consequently, nitrocellulose paper was used in the assays.

Dot blot assay. To select the optimal amount of antigen to be used in the assay, we checked several coating doses of glycolipid by testing a positive control serum with an OD of 0.8 by the ELISA and a negative serum with an OD of <0.1. We considered the optimal coating dose to be the amount that gave a clear signal for the positive control but that remained negative for sera derived from healthy individuals. Among the several amounts of antigen tested, 1 μg of DAT per spot showed the best results. With smaller amounts very weak signals were obtained.

To detect the antigen-antibody reaction we began by using a protein A-gold conjugate immediately after incubation with sera. However, the signal observed was weak and not very clear, particularly with those sera with low ODs. To enhance the sensitivity of the assay, we used a second incubation step with a rabbit anti-human serum, which increased the signal significantly (Fig. 1), making it possible to detect sera with low antibody titers.

Having thus determined the optimal antigen coating dose, incubation periods, and detection system, we used this technique to assay sera from patients with tuberculosis and sera from healthy subjects as negative controls. The assay was done at least twice, and we scored as positive only those tests that gave clear signals. Table 1 shows the results of parallel testing of the sera by ampELISA, the indirect ELISA with β-galactosidase, and the dot blot assay described above. We observe that by the enhanced dot blot assay it was possible to detect sera with an OD of about 0.170. The intensities of the spots were generally proportional to the ODs in the ELISA. The cutoff
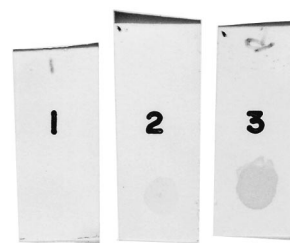

FIG. 1. Dot blot assay with DAT. The glycolipid was spotted onto each strip, and the strips were developed by the technique described in Materials and Methods. Strip 1, negative control serum; strip 2, positive serum developed with the protein A-gold conjugate only; strip 3, the same positive serum in strip 2 developed with an anti-human IgG serum raised in a rabbit, followed by incubation with the protein A-gold conjugate.

point for the ELISA was 0.174 and that for the ampELISA was 0.574 (2 standard deviations from the mean OD for the negative sera). The sensitivities were 50% for the dot blot assay, 47.73% for the ELISA, and 77.27% for the ampELISA. The specificities of these assays were 97.14, 94.29, and 97.14%, respectively. We did not observe a correlation between the mycobacterial load observed in Ziehl-Neelsen-stained smears, the chronicity of the disease, or the age of the patients with the OD observed in the ELISA or the ampELISA.

DISCUSSION

Several antigens of *M. tuberculosis* have been found to be useful in the serodiagnosis of tuberculosis. Among them, some glycolipids have been observed to be immunogenic, e.g., phenolglycolipid I (25), lipooligosaccharides (18), and DATs (7). ELISAs with DAT have shown good levels of sensitivity and specificity (2, 15), although in some instances low sensitivities have been observed (17, 19, 23).

Although the ELISA system is very practical and sensitive, the testing equipment required is not always available in areas where tuberculosis is endemic. A simple field testing method is therefore needed. An alternative to ELISA could be the dot blot method, which uses only a paper matrix onto which the antigen is spotted, and the development of the antigen-antibody reaction is done by an enzyme immunoassay or the use of a colloidal gold conjugate (9, 24).

By using a protein A-gold conjugate the system becomes very simple. However, when we tried this direct method, the antigen-antibody reaction with protein A-gold resulted in faint spots and therefore had a low sensitivity. Since the reaction with protein A-gold depends on the interaction of the Fc fragment of IgG with protein A, we used a second antibody against human immunoglobulins raised in rabbits, a species whose IgG also binds to protein A. By using this technique, the sensitivity of the dot blot assay was considerably increased, making it possible to detect a serum sample with an OD of about 0.200. There exists a good correlation between the OD obtained by ELISA and the intensity of the signal in the dot blot assay (Table 1). Although we expected the dot blot assay to have a lower sensitivity than the ELISA, the dot Blot assay detected the same number of positive patients as the indirect ELISA, suggesting a possible usefulness of the assay in field tests.

The sensitivities of ELISAs with sulfolipid IV (DAT) have been observed to vary from 25 to 74% in diverse studies (7, 23). This variation in sensitivities is probably due to the heterogeneity of the groups of patients analyzed, to small differences in the technique used, or to small differences in the distribution of the immunodominant glycolipids in the different batches of

TABLE 1. Clinical data for patients whose sera were studied and results of dot blot assay, ELISA, and ampELISA[a]

Serum sample no.	Patient age (yr)	Diagnosis[b]	Smear result	Culture result	Dot blot assay result	OD ELISA	OD ampELISA
1	37	Pulm. Tb.	++	+	−	0.000	0.455
2	39	Pulm. Tb.	+	+	−	0.024	0.814
3	26	Pulm. Tb.	+++	+	−	0.032	1.530
4	40	Pulm. Tb.	+++	+	−	0.040	0.724
5	38	Pulm. Tb.	++	+	−	0.045	0.460
6	57	Pulm. Tb.	+	+	±	0.050	1.546
7	36	Pulm. Tb.	+++	+	−	0.055	0.287
8	37	Pulm. Tb.	+	+	−	0.056	0.218
9	50	Pulm. Tb.	++	+	±	0.058	1.345
10	19	Pulm. Tb.	+	+	−	0.067	0.230
11	36	Pulm. Tb.	+	+	−	0.070	0.482
12	43	Pulm. Tb.	++	+	±	0.080	1.032
13	69	Pulm. Tb.	++	+	−	0.084	0.845
14	37	Pulm. and Pleural Tb.	++	ND[c]	+	0.085	0.906
15	42	Pleural Tb.	+	ND	−	0.088	0.928
16	64	Pulm. Tb.	++	+	−	0.096	0.689
17	26	Pulm. Tb.	+++	+	−	0.100	1.185
18	41	Pulm. Tb.	+	+	−	0.103	0.641
19	52	Pulm. Tb.	+	ND	−	0.112	0.571
20	61	Pulm. Tb.	++	+	+	0.117	0.958
21	36	Pulm. Tb.	+	+	−	0.156	0.849
22	21	Pulm. Tb.	++	+	−	0.160	0.893
23	78	Pulm. Tb.	+	+	−	0.161	0.401
24	38	Pulm. Tb.	+	+	+	0.179	2.227
25	81	Pulm. Tb.	+	+	+	0.190	1.744
26	54	Pulm. Tb.	++	+	++	0.232	1.998
27	32	Pulm. Tb.	+	+	+	0.233	2.054
28	16	Peritoneal Tb.	+	ND	+	0.252	1.063
29	42	Pulm. Tb.	+	+	−	0.285	0.448
30	61	Pulm. Tb.	+	ND	+	0.290	2.035
31	38	Pulm. Tb.	++	+	+	0.352	1.973
32	66	Pulm. Tb.	+	+	++	0.357	0.738
33	47	Pulm. Tb.	+++	+	++	0.421	1.379
34	40	Pulm. Tb.	+	+	+	0.477	2.161
35	78	Pulm. Tb.	+++	+	++	0.542	2.238
36	63	Pulm. Tb.	+++	+	++	0.667	2.121
37	54	Pulm. Tb.	++	+	++	0.675	2.147
38	24	Pulm. Tb.	++	+	++	0.706	2.348
39	18	Pulm. Tb.	+	+	++	0.759	2.128
40	46	Pulm. Tb.	++	+	++	0.824	1.903
41	56	Pulm. Tb.	++	+	++	0.936	2.500
42	55	Pulm. Tb.	++	+	+++	1.231	2.215
43	62	Pulm. Tb.	+++	+	+++	1.234	2.163
44	50	Pulm. Tb.	++	+	++	1.256	2.548

[a] The underlined ODs for the results of the ELISA and the ampELISA and the results designated ± for the dot blot assay were considered negative results.
[b] Pulm., pulmonary; Tb., tuberculosis.
[c] ND, not determined.

DAT used, since it has been observed that DAT is a mixture of several chemical species (1, 3). The differences in sensitivity can also be explained by the person-to-person variation in the host immune response to *M. tuberculosis* antigens. It has been observed that patients' sera react differently to several antigens, with antibodies detected in only 25 to 44% of the patients when single antigens are tested (16). However, when a panel of antigens is used, it is possible to detect almost the 90% of the cases of infection (16). It is possible that the use in tandem of other *M. tuberculosis* immunodominant antigens of a different chemical nature (e.g., proteins and polysaccharides) can increase the level of detection of positive patients. An assay of this kind would help to diagnose infection in those patients for whom microscopic examination is negative or is not easy to perform, such as pediatric patients with tuberculosis or patients with extrapulmonary disease (disease in the meninges, bones, kidneys, etc).

In the present work the sera were also analyzed with an ampELISA system, a more sensitive method, which was developed on the basis of a previously reported system (10, 11). This technique uses an enzymatic method that recycles the NAD obtained by hydrolysis of NADP by the alkaline phosphatase, and it has been claimed to be able to detect 0.01 amol of alkaline phosphatase (10, 11). By using this system it was possible to increase the sensitivity to 77.27%, detecting antibodies in 35 of 44 patients tested. Among the negative patients, some of the patients (six of nine) had had symptoms for 2 months or less. It is possible that measurable responses need longer periods of time to develop.

The goal of this work was to develop a simple test that could

replace the ELISA system for use at locations where the appropriate equipment is not easily available. Although the dot blot assay reached a sensitivity similar to that of ELISA, a more sensitive method of detection is needed. Although the ampELISA showed good sensitivity, it is technically complex. The development of an amplification system in a solid enzyme immunoassay system, catalyzed reporter deposition, that yielded an 8- to 200-fold increase in sensitivity over that of a called conventional solid-phase enzyme immunoassay has been reported previously (4). This method is simple in theory and could be a good candidate for a dot blot system in field trials for the diagnosis of tuberculosis.

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